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TITLE: DNA Cleavage/Repair and Signal Transduction Pathways in Irradiated Breast Tumor Cells

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To whom it may concern:

We would like to provide this addendum which indicates the work that has been accomplished in our laboratory during this past year of support.

Our studies have been extended primarily in two areas, which are described in some detail below.

1. Selective enhancement of radiation sensitivity in the breast tumor cell by analogs of Vitamin D_3.

Rationale: One primary limitation to the utilization of radiotherapy in the treatment of breast cancer is the toxicity of radiation (both immediate and delayed) to normal host tissue. Consequently, the identification of approaches to enhance the effectiveness of low doses of radiation without concomitant increases in host tissue toxicity (i.e. a favorable therapeutic ratio), could improve the clinical treatment of breast cancer patients with fractionated radiation.

Current Findings: We have pursued this concept by demonstrating that two lead analogs of Vitamin D_3, EB 1089 and ILX-2307553, can promote radiation sensitivity in the breast tumor cell, apparently through the promotion of apoptotic cell death. Figure 1 indicates that the decline in clonogenic survival after treatment of MCF-7 breast tumor cells with the combination of EB 1089 and fractionated radiation (5 x 10 Gy) exceeds the reductions produced by exposure to either modality alone; furthermore, the effect of the combination on clonogenic survival is clearly greater than additive. We believe that these findings are related to the promotion of apoptotic cell death by EB 1089 in combination with fractionated radiation. Table I presents the results of the alkaline unwinding assay which reflects DNA fragmentation; the increase in radiation equivalence of DNA damage induced by EB 1089 + fractionated radiation over that produced by either irradiation alone (essentially baseline levels) or EB 1089 alone (minimal levels) is indicative of enhanced apoptosis. These findings that EB 1089 promotes apoptosis in response to fractionated radiation have been confirmed using the TUNEL assay for DNA fragmentation (not shown).

Perhaps of greatest interest is the finding that EB 1089 as well as ILX23-7553 (data with ILX-23-

7553 not shown) fail to enhance the response of human BJ fibroblasts to fractionated radiation in a cell viability assay (Figure 2). In fact , the effect of the combination of EB 1089 with fractionated radiation on cell viability was clearly less than additive as the combined effect did not exceed the reduction in cell viability produced by either modality alone. Clonogenic analysis was not feasible as the BJ fibroblasts fail to form colonies under normal cell culturing protocols; however, our previous work examining the combination of Vitamin D_3analogs with either adriamycin or radiation has demonstrated that the cell viability assay is predictive of clonogenic survival. A similar (less than additive) effect of the combination of EB 1089 with fractionated radiation was observed in normal human breast epithelial cells (not shown).

Implications: It is important to reiterate that clinical radiotherapy protocols for breast cancer involve multiple radiation doses of 2 Gy (consistent with the approach utilized in our current work). Our findings suggest that the Vitamin D_3 analogs which are being developed to circumvent the toxicity associated with elevated doses of Vitamin D_3 may have the potential to enhance the sensitivity of breast tumor cells to fractionated radiation without increasing toxicity to normal tissue.

Future Studies: We are currently performing or planning the following studies to build upon these findings:

- combining EB 1089 with fractionated radiation in human breast tumor cell xenografts;
- developing approaches to identify the basis for conferring susceptibility to apoptosis by the Vitamin D_3 analogs in the breast tumor cell;
- attempting to understand the putative role of p53 in the response of the breast tumor cell to Vitamin D_3 analogs in combination with fractionated radiation;
- assessing the basis for the apparent selectivity (and positive therapeutic ratio) of the Vitamin D_3 analogs in conferring sensitivity to radiation in the breast tumor cell.

2. Senescence and recovery after radiation in the breast tumor cell.

Rationale: Although radiation therapy is effective at shrinking tumors in anticipation of surgery and ostensibly eliminating breast cancer cells, many patients ultimately suffer from disease recurrence. We hypothesize that this recurrence may be related, at least in part, to the existence of a few residual cells which lie dormant or quiescent for an extended time period, but ultimately recover proliferative capacity. There have been some hints in the literature that this recovery may occur in studies of repopulation after irradiation. Consequently, we have initiated studies to determine whether a subpopulation of breast tumor cells survives and recovers reproductive function after irradiation, and to investigate the biochemical and molecular characteristics of the recovered cells.

Current Findings: This work has led to the observations that irradiation of p53 wild-type MCF-7 and ZR-75-1 breast tumor cells results in an apparent senescence response while irradiation of p53 mutant MDA-MB231 cells does not result in senescence but rather in a delayed apoptotic cell death (these findings appeared in our final report). In more recent work, we have determined that a small fraction of the cell population recovers proliferative function, but only after a long delay (on the order of 12-14 days). We have linked the senescence response to down-regulation of telomerase activity; furthermore, preliminary studies suggest that cells that recover proliferative capacity also

recover telomerase function. Finally, in even more preliminary studies, we have the suggestion that treatment with the Vitamin D_3 analogs which promote apoptosis in response to radiation delays the recovery of reproductive function in the breast tumor cell.

Implications: These studies suggest that:
- senescence may be permissive for ultimate recovery of tumor cell growth after irradiation;
- recovery of reproductive capacity may be linked to telomerase activity;
- promotion of apoptosis could influence (at least delay but perhaps abrogate) recovery of the tumor cells.

Future Studies: We are currently pursuing these findings to address the questions indicated below:

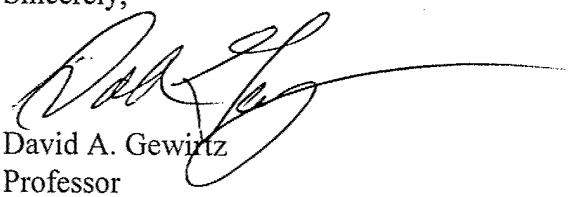
1. What are the characteristics of the cells which recover proliferative function? Are these cells resistant to radiation (or chemotherapeutic drugs) or might these cells simply represent a subpopulation which evaded the initial injury?
2. Might recovery be associated with the capacity of the cells to repair sublethal damage?
3. What molecular pathways are involved in regulation of DNA repair and recovery?
4. Do the Vitamin D_3 analogs alter DNA repair or is their effect on recovery limited to promotion of apoptosis?
5. Is the prolonged senescence response related to breaks in the telomere?

Two manuscripts are currently in preparation relating to the studies involving Vitamin D_3 analogs in combination with irradiation and an additional two manuscripts are in preparation relating to the tumor cell responses of senescence and recovery.

We have submitted a proposal related to DNA repair and recovery to the recent Breast Cancer Research Program sponsored by the Department of Defense and are planning on submitting two proposals to the NIH relating to the research areas described above.

If additional information is required, my contact information is presented below.

Sincerely,



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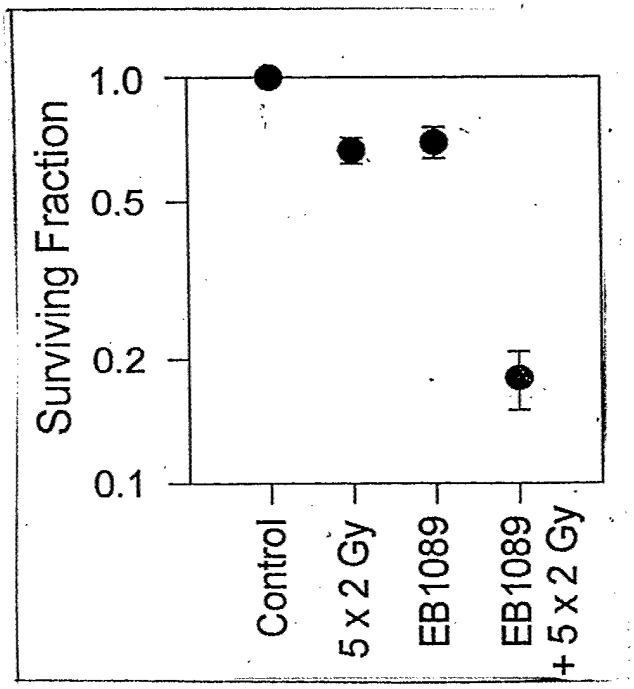

Figure 1. Influence of EB 1089 on the clonogenic survival of MCF-7 breast tumor cells. Cell were treated with EB 1089 (100nM) for 72 hours and replaced with fresh media prior to fractionated radiation (2 x 5 Gy) . Following the fifth dose of radiation, cells were plated at different densities and colony formation was assessed after 12 days. Data represent means +/- range from two independent experiments.

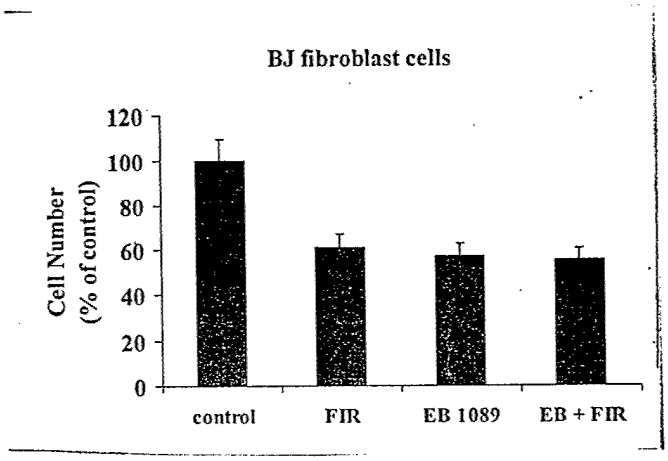

Figure 2. Influence of EB 1089 on the antiproliferative effects of fractionated radiation in BJ fibroblasts cells. Cell were treated with EB 1089 (100nM) for 72 hours and replaced with fresh media prior to fractionated radiation (2 x 5 Gy) . Following the fifth dose of radiation, viable cell number was determined by trypan blue exclusion. Data represent means +/- range from two independent experiments.

Table I. Alkaline unwinding assay in MCF-7 cells. Cells were treated with 100nM EB 1089 for 72 hr. After 72 hours, EB 1089 was removed and cells were irradiated. Following the last irradiation dose, cells were prepared for the alkaline unwinding assay which assessed DNA fragmentation in terms of rad equivalence of DNA damage. Data represent means +/- range from two independent experiments.

Treatment	**RAD Equivalents**
Control	96 ± 2
EB 1089	187 ± 4
IR (5x 2Gy)	100 ± 3
EB1089 + IR	1082 ± 8